*Proc. Natl. Acad. Sci. USA*
Vol. 79, pp. 2589–2593, April 1982
Cell Biology

# Establishment of a cell line from lepidopteran wing imaginal discs: Induction of newly synthesized proteins by 20-hydroxyecdysone

(two-dimensional electrophoresis/*Trichoplusia ni*)

D. E. LYNN*, S. G. MILLER*, AND H. OBERLANDER†‡

*Department of Entomology, University of Florida, Gainesville, Florida 32611; and †Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agriculture Research Service, United States Department of Agriculture, Gainesville, Florida 32604

*Communicated by Howard A. Schneiderman, December 7, 1981*

ABSTRACT **A cell line has been developed from the imaginal wing discs of the lepidopteran insect *Trichoplusia ni* (Hübner). The cells grow in suspension as fluid-filled, multicellular vesicles. The cell line has been designated IAL-TND1. Two-dimensional electrophoresis (isoelectric focusing and sodium dodecyl sulfate/polyacrylamide gels) of [$^{35}$S]methionine-labeled proteins revealed similar patterns for IAL-TND1 cells and whole *T. ni* wing imaginal discs. Additionally, exposure to the insect steroid hormone 20-hydroxyecdysone stimulated the synthesis of the same new proteins in both whole wing discs and IAL-TND1 cell vesicles.**

Numerous studies of morphogenesis and hormonal action in insects have focused on the imaginal discs as a model target tissue (1, 2). Imaginal discs are undifferentiated but determined primordia that are assembled into organized packets of cells in immature holometabolous insects such as Diptera and Lepidoptera. These primordia are destined to become adult structures such as legs, wings, and genitalia during metamorphosis which is stimulated by an increased titer of 20-hydroxyecdysone and a decreased titer of juvenile hormone.

Recently, continuous insect cell cultures have shown much promise in studies of developmental biology (3–6). Many insect cell lines have been developed [more than 176 cell lines from 58 species (7, 8)], but these were often intended for use in pathology so that little concern was taken to determine the developmental state or type of tissue that was selected. Manifestly, it would be a considerable aid to developmental and hormonal studies with insect cells if a cell line could be established from imaginal discs. We agree with Echalier (9) that we need to "learn how to grow *in vitro* every type of differentiated insect cells and that such cells continue to perform their complete specialized programs." Our collective experience with imaginal discs and cell lines of various Lepidoptera led us to attempt to develop cell cultures from the wing imaginal discs of Lepidoptera. These discs are relatively easy to isolate from the larvae and have proven useful in morphological and biochemical studies of insect development (10–12). *Trichoplusia ni* (Hübner) has been the source of important cell lines derived from ovaries and fat body (8) and we reasoned that the imaginal discs of this species might be less refractory to cell culture than some others.

In this communication we report the successful establishment of a continuous cell line derived from wing imaginal discs of last-instar larvae of *T. ni*.

## MATERIALS AND METHODS

*T. ni* in the last larval instar (150–200 mg) were obtained from laboratory colonies. Individuals were surface sterilized (70% ethanol, 15 min) and imaginal wing discs were explanted as described (13). After meticulous removal of extraneous tissues (primarily fat body) and rinsing of the wing discs in three changes of culture medium [Yunker *et al.* modification (14) of Grace's medium (15) containing 10% fetal bovine serum (GIBCO)], the discs (20 discs for each of four cultures) were transferred to a standing drop (0.2 ml) of medium in a 35-mm plastic tissue culture Petri dish (Falcon). Each disc was cut into four to eight pieces and the culture was incubated at 26°C and 95% relative humidity. After 48 hr, an additional 1.0 ml of medium was added to the culture. Half of the medium was replaced at 5- to 10-day intervals until the first subculture, which was made on one culture after 10 wk in culture. The subculture procedure involved rupturing the vesicles by gentle pipetting, centrifuging at 40 × *g* for 5 min, and resuspending the cells in 0.5 ml of old medium and 4.0 ml of fresh culture medium in a Costar 25-cm$^2$ tissue culture flask. Subcultures were made at weekly intervals at 1:2 to 1:4 dilution ratios.

The chromosomes of the resulting cell line were examined by fixing and staining cultures according to the procedure of Earley (16). Counts were made on 50 randomly chosen chromosome spreads at the 5th and 27th passage levels.

Comparison of protein synthesis in whole *T. ni* imaginal discs, fat body, and hemocytes with those of the cell cultures was made by utilizing two-dimensional polyacrylamide gel electrophoresis. Imaginal discs, hemocytes, and fat body were obtained from *T. ni* larvae as described above except that the discs were left whole in culture medium (five discs per 1 ml of medium) rather than being cut into pieces. After 3 days in culture, the tissues were transferred to 1 ml of Grace's medium (15) that lacked methionine and contained 50 μCi of [$^{35}$S]methionine (1050.5 Ci/mmol; 1 Ci = 3.7 × 10$^{10}$ becquerels; New England Nuclear) and were incubated for 5 hr at 27°C. Cultures of IAL-TND1 vesicles were also replenished with 1 ml of methionine-free Grace's medium that contained 50 μCi of [$^{35}$S]methionine and incubated for 5 hr at 27°C.

Tissues and cells were homogenized with micro tissue grinders in the lysis buffer of Rogers and Shearn (17), and isoelectric focusing was performed in tube gels over the pH range of O'Farrell (18). Second-dimension electrophoresis was in $NaDodSO_4$/12.5% polyacrylamide gels; then, the gels were fixed, dried, and exposed to Kodak BB-5 x-ray film for periods up to 2 weeks as described (19). Similar cultures of imaginal discs and vesicular cells were exposed to 1 μM 20-hydroxyecdysone for 24 and 48 hr prior to labeling with [$^{35}$S]methionine and electrophoresis. Each gel was loaded with homogenized material containing 5 × 10$^5$ cpm.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

‡ To whom reprint requests should be addressed.

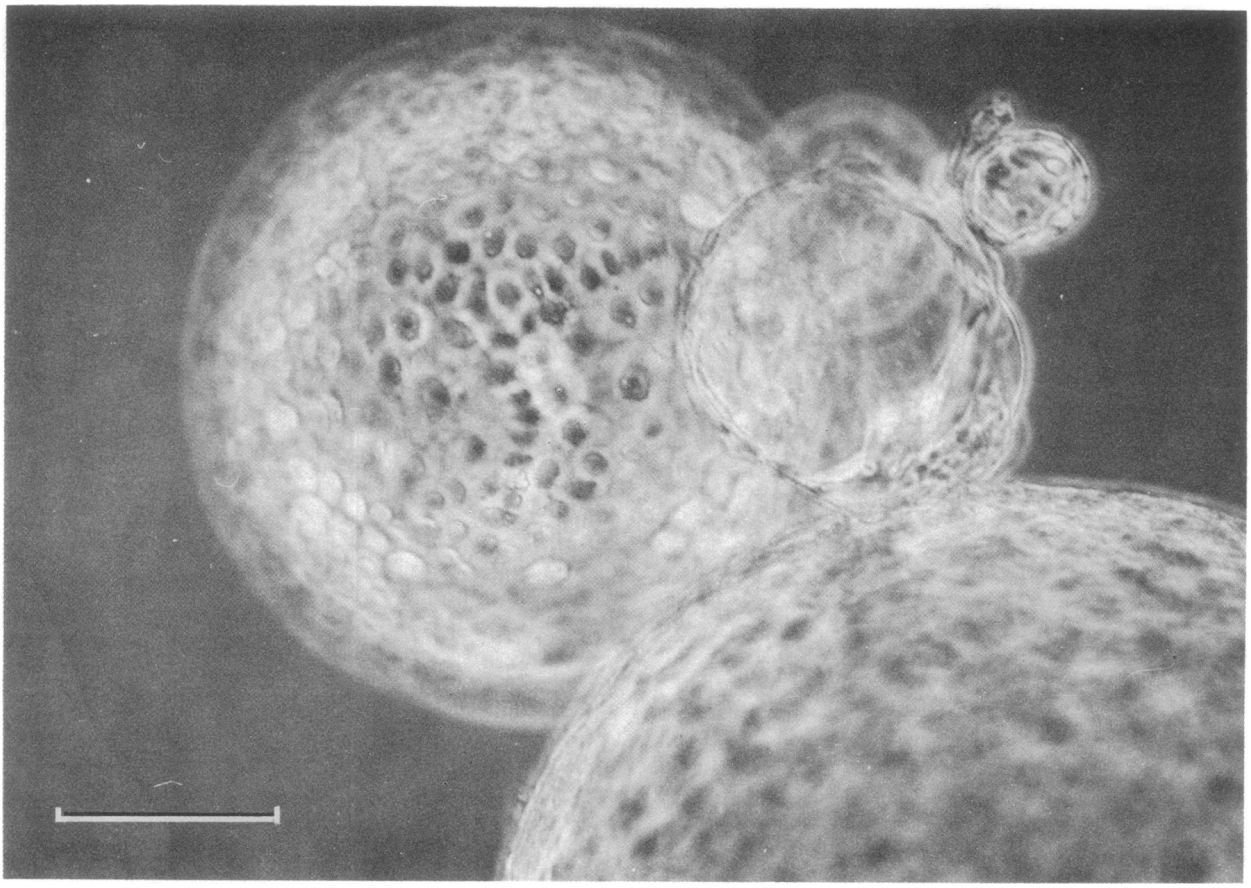

FIG. 1. Phase-contrast micrograph of IAL-TND1 cellular vesicles at the third passage *in vitro*. (Bar equals 100 $\mu$m.)

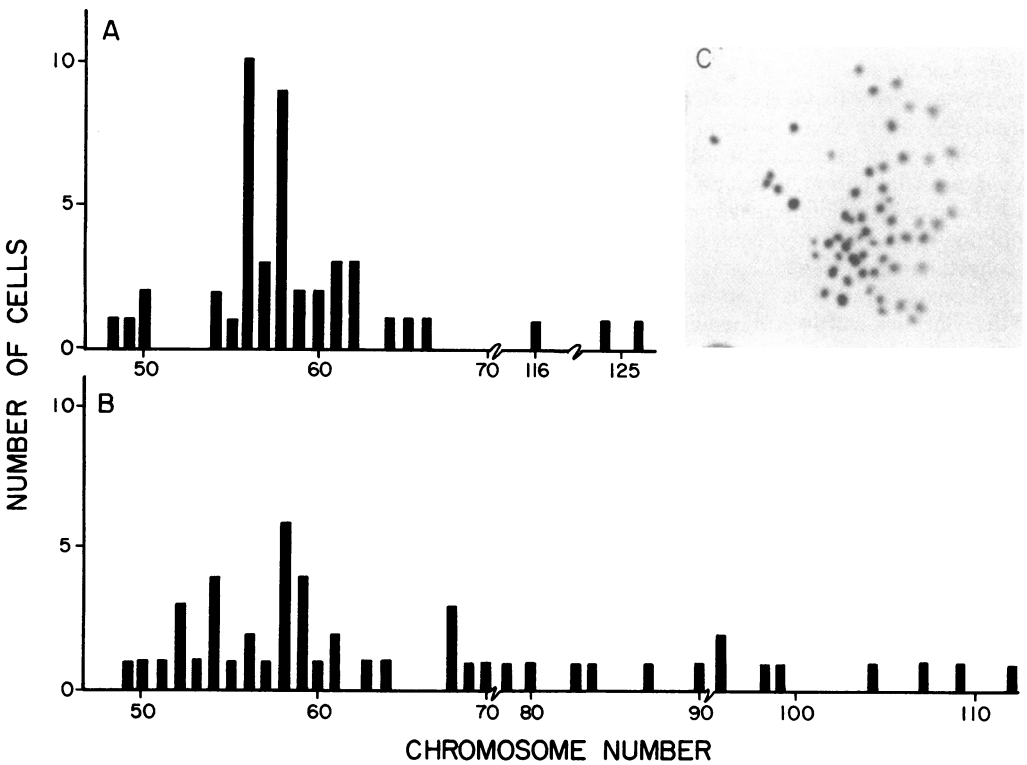


FIG. 2. Chromosome counts on 50 cells at the 5th (*A*) and 27th (*B*) passages. (*C*) Photomicrograph of chromosome spread at 27th passage.

## RESULTS

From the initial four cultures of *T. ni* imaginal wing discs, one proved more rapidly growing and was used to develop the continuous cell line described in this paper. Within 48 hr in culture, multicellular blebs formed on the wing disc fragments and monolayer outgrowths occurred on the surface of the culture. At approximately 1-wk intervals, the vesicular blebs were recut with a scalpel in an attempt to maintain the cells as monolayers. At the time of the first subculture, the monolayers re-formed free-floating vesicles and subsequently were maintained in that form by using the subculture technique described above. The cell line has been designated IAL-TND1 and its appearance is shown in Fig. 1. The cells typically occurred in suspension as multicellular, fluid-filled vesicles consisting of a monolayer of epithelium-like cells. After approximately 35 passages, the cultures began to undergo spontaneous, but reversible, morphological changes to solid aggregates of round cells. Currently, the IAL-TND1 cultures generally consist of approximately 30% vesicles and 70% clumped cells. We believe this morphological variability to be the result of suboptimal culture conditions.

Chromosome analysis showed small chromosomes that are typical of Lepidoptera. At low passage numbers, the cultures were primarily nearly diploid (mode = 56; range 49–126; *in vivo* haploid number = 31). At higher passages, the number of cells near triploid and tetraploid increased, although many cells were still near diploid numbers (Fig. 2). This relative stability in chromosome numbers is somewhat unusual for lepidopteran cell cultures which frequently are highly polyploid and have greater variability in chromosome numbers than we have seen with IAL-TND1 (7, 8).

Comparison of two-dimensional electrophoretic patterns of [$^{35}$S]methionine-labeled proteins in IAL-TND1 vesicles and whole *T. ni* imaginal wing discs revealed similar patterns of protein synthesis (Fig. 3 *A* and *B*) with only slight quantitative variation apparent. Additionally, treatment with physiological levels of 20-hydroxyecdysone (1 $\mu$M) stimulated the synthesis of at least three new proteins in vesicles after 30 wk in culture (Fig. 3*C*). The same new proteins were induced in freshly explanted whole wing discs (Fig. 3*D*). In addition, three proteins present in control cultures of discs and cell cultures were no longer synthesized in hormone-treated cultures. There were no apparent qualitative differences between cultures treated with 20-hydroxyecdysone for 24 or 48 hr, although some quantitative differences may exist. Our results indicate that the IAL-TND1 cells are indeed imaginal disc cells because electrophoretic patterns of other tissues, including hemocytes (Fig. 4*A*), fat body (Fig. 4*B*), and midgut, differed conspicuously from both whole wing discs and vesicle cells. Additionally, the induction of appropriate new proteins after incubation with 20-hydroxyecdy-

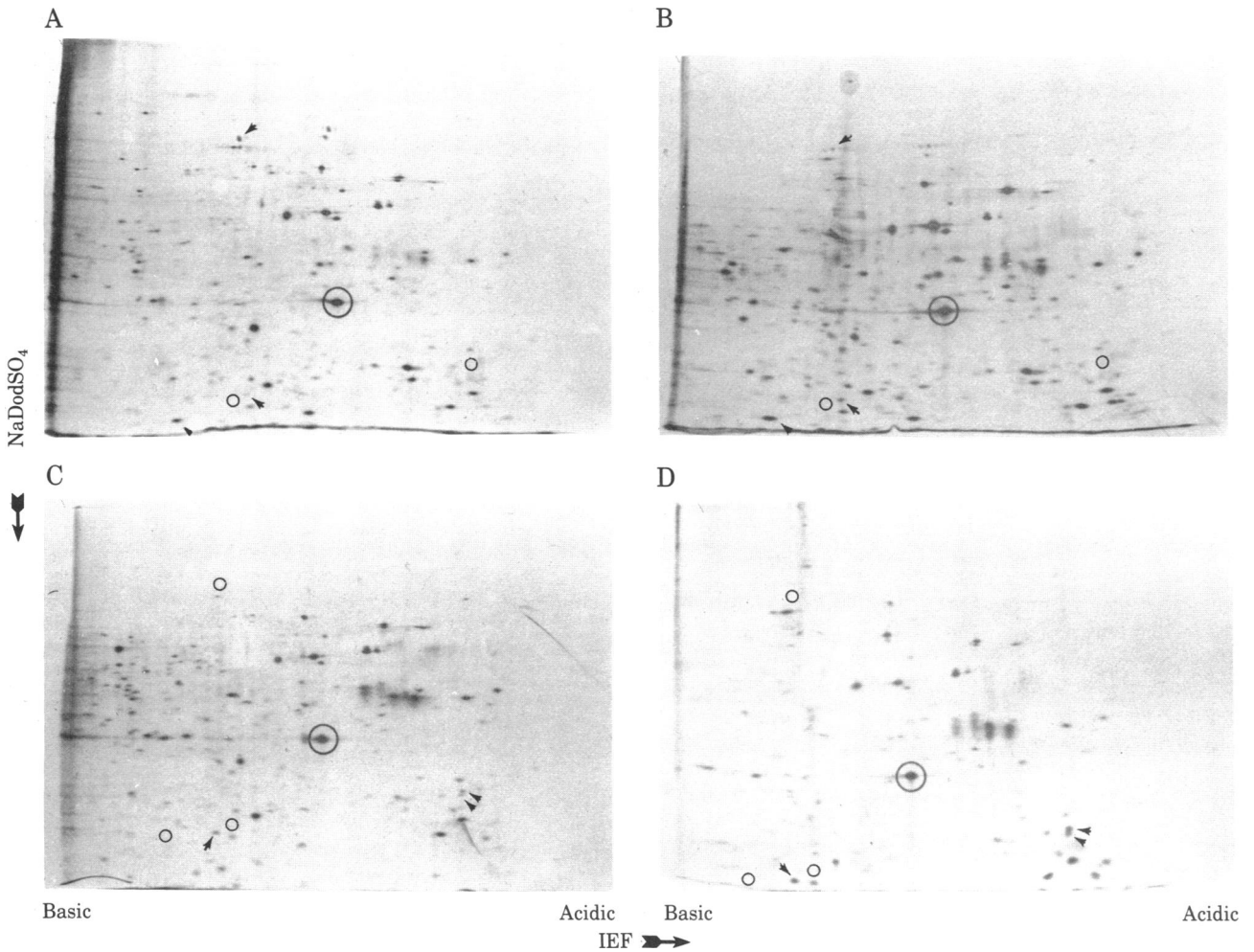


FIG. 3. Autoradiographs of [$^{35}$S]methionine-labeled proteins after isoelectric focusing (IEF) and NaDodSO$_4$ electrophoresis on 12.5% acrylamide gels. Arrows and small circles indicate locations of the polypeptides that vary in response to 20-hydroxyecdysone. The large circle indicates cytoplasmic actin used as a landmark in the gels based on the location of an actin standard. (*A*) IAL-TND1 cells; (*B*) *T. ni* wing disc; (*C*) IAL-TND1 cells after 24-hr exposure to 1 $\mu$M 20-hydroxyecdysone; (*D*) *T. ni* wing discs after 24-hr exposure to 1 $\mu$M 20-hydroxyecdysone.

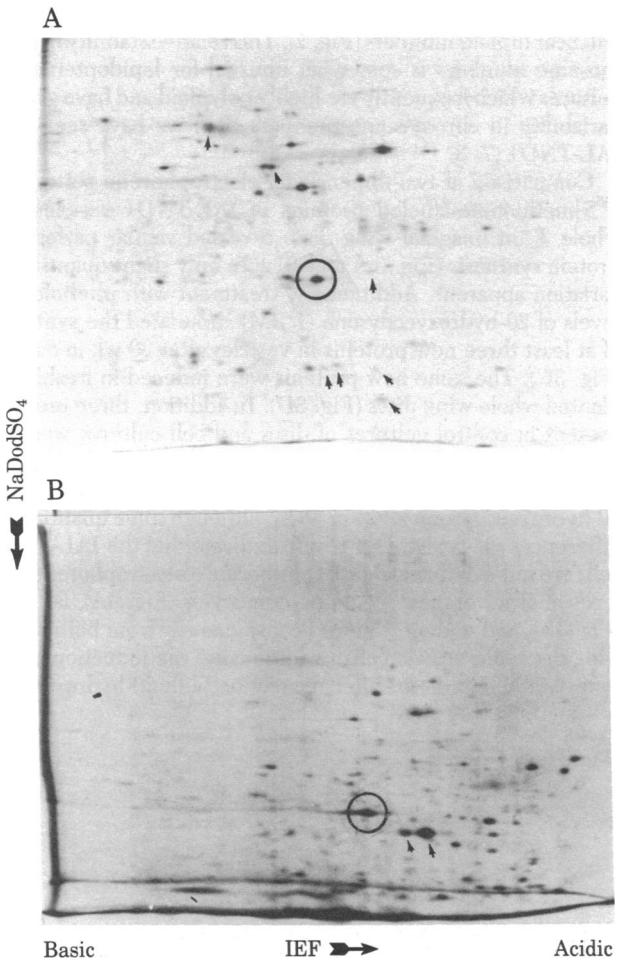


FIG. 4. Autoradiographs of two-dimensional electrophoresis gels as in Fig. 3. (*A*) [$^{35}$S]Methionine-labeled proteins from cultured *T. ni* hemocytes; (*B*) [$^{35}$S]methionine-labeled proteins from cultured *T. ni* fat body. Arrows indicate examples of proteins not found in imaginal wing discs.

sone indicates that the IAL-TND1 cells retain their ability to differentiate in response to hormonal cues, even after 30 wk in culture. Moreover, hormonal induction of new proteins continued to be observed in cultures that grow primarily as clumped cells rather than as vesicles.

## DISCUSSION

We have obtained growth of cells in continuous culture from imaginal wing discs of the lepidopteran *T. ni*. This cell line is remarkable in several aspects. (*i*) It is developed specifically from imaginal discs and is one of the few insect cell lines that appears to consist uniformly of one tissue type (epithelial). (*ii*) This cell line maintains the ability to differentiate with respect to new protein synthesis when exposed to the insect steroid hormone, 20-hydroxyecdysone. (*iii*) The cultures maintain a relatively high level of organization by growing as multicellular vesicles.

Previously, insect cell cultures were established by using embryos, ovarian sheaths, hemocytes, or macerated young larvae. Each of these tissue sources consists of heterogeneous cell types in varied developmental states so that the resulting cell cultures are only of general use in studies of insect development (9). Alternatively, our culturing conditions with imaginal disc cells provides a homogeneous population of epithelium-like cells obtained from tissues in a known developmental state. Although previous attempts at culturing imaginal discs showed that growth was possible with these tissues, continuous cell lines were not established (20, 21). The cell line developed in our studies should be useful in studying various developmental processes, including studies on hormone receptors and gene action. The cell line may be appropriate for research on the maintenance of determination, although at present we can only state with certainty that an ability to respond to hormones is maintained because recognizable wing structures were not made *in vitro*.

Induction of new peptides in response to ecdysteroids has been reported with *Drosophila* cell cultures (22–25), but it was not possible to correlate the new proteins with changes in specific tissues because these cells are from embryos and are of unknown cell type. However, the IAL-TND1 line was established from a specific tissue source, and we were able to correlate the peptide changes to similar changes in the intact wing discs.

The high level of organization apparent in these cell cultures could broaden their use in developmental studies. IAL-TND1 cell cultures should be useful in investigations of epithelial transport and cytoskeletal problems that involve maintenance of the vesicular shape as well as developmental studies on intercellular transport and communication. The formation of vesicles *in vitro* is not unique with our system. There are also primary cultures of insect tissues (26, 27), and two continuous insect cell lines that form vesicles (28, 29). Also, a line of mammalian epithelial cells forms domes (30) which are structurally similar to vesicles. However, the IAL-TND1 cells provide a special opportunity for examining hormonal action and morphogenesis in a continuous line of insect cells derived from imaginal discs with a known developmental history.

We thank C. E. Leach for technical assistance and express our appreciation to J. H. Willis, G. Bergtrom, and J. Vaughn for reviewing the manuscript. D.E.L. and S.G.M. were Postdoctoral Fellows employed through a cooperative agreement between the University of Florida and the Insect Attractants, Behavior, and Basic Biology Research Laboratory.

1. Silvert, D. J. & Fristrom, J. W. (1980) *Insect Biochem.* **10**, 341–355.
2. Ursprung, H. & Nöthiger, R., eds. (1972) *The Biology of Imaginal Disks* (Springer, New York).
3. Cherbas, L., Cherbas, P., Savakis, C., Demetri, G., Manteuffel-Cymborowska, M., Yonge, C. D. & Williams, C. M. (1980) in *Invertebrate Systems in Vitro*, eds. Kurstak, E., Maramorosch, K. & Dübendorfer, A. (Elsevier/North-Holland, Amsterdam), pp. 217–228.
4. Marks, E. P. & Holman, G. M. (1979) *In Vitro* **15**, 300–307.
5. Lynn, D. E. & Oberlander, H. (1981) *Roux's Arch. Dev. Biol.* **190**, 150–155.
6. Kurstak, E., Maramorosch, K. & Dübendorfer, A., eds. (1980) *Invertebrate Systems in Vitro* (Elsevier/North-Holland, Amsterdam).
7. Hink, W. F. (1976) in *Invertebrate Tissue Culture, Research Applications*, ed. Maramorosch, K. (Academic, New York), pp. 319–368.
8. Hink, W. F. (1980) in *Invertebrate Systems in Vitro*, eds. Kurstak, E., Maramorosch, K. & Dübendorfer, A. (Elsevier/North-Holland, Amsterdam), pp. 553–578.
9. Echalier, G. (1980) in *Invertebrate Systems in Vitro*, eds. Kurstak, E., Maramorosch, K. & Dübendorfer, A. (Elsevier/North-Holland, Amsterdam), pp. 589–592.
10. Ferkovich, S. F., Oberlander, H., Leach, C. E. & Van Essen, F. (1980) in *Invertebrate Systems in Vitro*, eds. Kurstak, E., Maramorosch, K. & Dübendorfer, A. (Elsevier/North-Holland, Amsterdam), pp. 209–216.
11. Nardi, J. B. & Willis, J. H. (1979) *Dev. Biol.* **68**, 381–395.
12. Blais, C. & Lafont, R. (1980) *Wilhelm Roux's Arch.* **188**, 27–36.

13. Oberlander, H. & Leach, C. E. (1979) *Tissue Cult. Assoc. Man.* **5**, 993–996.
14. Yunker, C. E., Vaughn, J. L. & Cory, J. (1967) *Science* **155**, 1565–1566.
15. Grace, T. D. C. (1962) *Nature (London)* **195**, 788–789.
16. Earley, E. M. (1975) *Tissue Cult. Assoc. Man.* **1**, 31–35.
17. Rogers, M. E. & Shearn, A. (1977) *Cell* **12**, 915–921.
18. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021.
19. Miller, S. G. & Oberlander, H. (1981) *In Vitro* **17**, 689–694.
20. Kurtti, T. J. & Brooks, M. A. (1970) *J. Invertebr. Pathol.* **15**, 341–350.
21. Davis, K. T. & Shearn, A. (1977) *Science* **196**, 438–439.
22. Berger, E., Ringler, R., Alahiotis, S. & Frank, M. (1978) *Dev. Biol.* **62**, 498–511.
23. Cade-Treyer, D. & Munsch, N. (1980) *FEBS Lett.* **117**, 19–22.
24. Savakis, C., Demetri, G. & Cherbas, P. (1980) *Cell* **22**, 665–674.
25. Cherbas, P., Cherbas, L., Savakis, C. & Koehler, M. M. D. (1981) *Am. Zool.* **21**, 743–750.
26. Kambysellis, M. P. & Schneider, I. (1975) *Dev. Biol.* **44**, 198–203.
27. Dübendorfer, A., Shields, G. & Sang, J. H. (1975) *J. Embryol. Exp. Morphol.* **33**, 487–498.
28. Peleg, J. & Sharar, A. (1972) *Tissue & Cell* **4**, 55–62.
29. Kurtti, T. J. & Brooks, M. A. (1977) *In Vitro* **13**, 11–17.
30. Lever, J. E. (1979) *J. Supramol. Struct.* **12**, 259–272.